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Artigo

Composition and Larvicidal Activity of Essential Oil of *Eugenia candolleana* DC. (MYRTACEAE) against *Aedes aegypti*

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Composição e Atividade Larvicida do Óleo Essencial de *Eugenia candolleana* DC. (MYRTACEAE) contra o *Aedes aegypti*

Resumo: O mosquito *Aedes aegypti* é o vetor do vírus da dengue, Chikungunya e Zika. O aumento da resistência dos mosquitos a inseticidas comerciais prejudica programas de controle regulares; portanto, a prospecção química de espécies de plantas com propriedade larvicida tem sido uma alternativa para o controle deste vetor. O óleo essencial de *Eugenia candollena* DC foi obtido através do método de hidrodestilação, utilizando um aparelho tipo Clevenger modificado, sendo seus constituintes identificados utilizando a cromatografia a gás acoplada à espectrometria de massas e quantificados pela cromatografia gasosa. O óleo obtido foi rico em sesquiterpenos, demonstrando o β-elemeno (35,87 ± 0,13%) como componente majoritário. A mortalidade das larvas do *A. aegypti* foi de 100% em 24 h após o tratamento com o óleo na concentração de 0,50 $\mu g.\mu L^{-1}$ e a CL_{50} para este tempo foi estimado em 0,30 $\mu g.\mu L^{-1}$.

Palavras-chave: Larvicida; CG-EM; sesquiterpenos; CL_{50}; *Aedes*; CG-EM.

Abstract

The mosquito *Aedes aegypti* is the vector of the dengue, Chikungunya and Zika virus. The increasing resistance of mosquitoes to commercial insecticides impairs regular control programs; therefore, chemical prospecting of plant species with larvicidal properties has been an alternative for the control of this vector. The essential oil of *Eugenia candollena* DC was obtained through the hydrodistillation method, using a modified Clevenger-type apparatus, being its constituents identified using gas chromatography-mass spectrometry and its constituents were quantified by gas chromatography. The oil proved rich in sesquiterpenes, containing β-elemene (35.87 ± 0.13%) as major component. *Aedes aegypti* larvae mortality rate of 100% was obtained after 24 h of treatment with the oil at concentrations of 0.50 $\mu g.\mu L^{-1}$ and the LC_{50} at this time was estimated to be 0.30 $\mu g.\mu L^{-1}$.

Keywords: Larvicidal; GC-MS; Sesquiterpenes; LC_{50}; *Aedes aegypti*.

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Composition and Larvicidal Activity of Essential Oil of *Eugenia candolleana* DC. (MYRTACEAE) against *Aedes aegypti*

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1. Introduction

The mosquito *Aedes aegypti* L. (Diptera: Culicidae) acts as a vector for various viruses, such as dengue, yellow fever, Chikungunya and the newcomer Zika, being considered by the World Health Organization as one of the major public health problems in the world.[1,2] The most widely adopted strategy to reduce the incidence of these diseases is to control the population of mosquito larvae. In view of the damage caused by dengue in the country, it is considered of paramount importance to find new methods to combat this vector. Plants naturally present defense mechanisms; therefore, material extracted from plant species has traditionally been used for controlling pest species of insects.[3-5] The control activities have detected mosquito resistance to chemical insecticides; therefore, the use of essential oils and plant extracts is gaining prominence as an alternative means of control of insect vectors. Additionally, they are not harmful to the environment.[6]

Essential oils consist of special volatile metabolites characterized by a strong odor and generally have a lower density than water. Oils are lipophilic in nature with a proven ability to interfere with metabolic, biochemical, physiological and behavioral functions in insects.[7]

The Myrtaceae family has been recognized for investigations regarding the chemical composition of their species and biological activities, mainly due to the presence of essential oils, making *Eugenia candolleana* DC a potential plant in larvicidal control. *Eugenia candolleana* DC., popularly known as "murta", "murtinha", "ameixa-da-mata" and "cereja-roxa" is a rare species of the *Eugenia* genus frequently found in Brazilian rainforests of the northwest, whose ripening fruit presents a dark purple appearance, with a slightly sweet and firm flesh. There are few studies related to this species in the literature,[8,9] although the infusion of its leaves has been popularly used to treat pain and fever.[9]

Therefore, the purpose of this study was to evaluate the chemical characterization and the ability of the essential oil of *E. candolleana* DC. to control *Aedes aegypti*.

2. Experimental

2.1. Plant material

Eugenia candolleana leaves were collected in the morning in the city of Seropédica - RJ, Brazil, Campus of the Universidade Federal Rural do Rio de Janeiro (UFRRJ) in July 2014, and identified by the Forest Engineer, Gabriel Pinto Rodrigues Gonçalves. A voucher specimen of the plant is placed in the herbarium RBR UFRRJ under RBR n° 36910.

2.2. Essential oil extraction

Fresh leaves (300.00 ± 0.15 g) of *E. candolleana* were subjected to hydrodistillation for 2 h using a modified Clevenger apparatus, the dimensions used in the equipment used do not interfere with the reproducibility of the data. Leaves were crushed and deposited in the distillation flask (6 L) along with 3500 mL of distilled water, and when the process was completed, the essential oil was separated from the water by decanting and dried with anhydrous sodium sulfate. The extracted essential oils were packed in an amber glass bottle at 4 °C and protected from light. The essential oil yield was calculated and expressed in weight of oil per weight of fresh leaves. The analyses were performed in triplicate.

2.3. Gas chromatography analysis

Quantitative analyses were performed using a chromatograph with gas equipped with a flame ionization detector (GC-FID) using a HP-5890 series II apparatus. The analyses were performed with a capillary column Factor Four-VF-5MS (30 m x 0.25 nm in diameter × 0.25 mm film thickness); at temperature programming 60 °C to 260 °C (3 °C.min^{-1}), then 10 °C to 290 °C.min^{-1}; gun temperature of 220 °C; interface temperature of 310 °C and detector of 280 °C. Helium was used as carrier with a flow rate of 1.0 mL.min^{-1}, split ratio 1:30 and 1.0 μL, the injected essential oil was diluted in dichloromethane. The analyses were performed in triplicate. Results were subjected to descriptive statistical analysis using the Statistical Analysis Software program.[10]

2.4. Gas chromatography-mass spectrometry analysis

Qualitative analysis was performed using a gas chromatography system coupled to mass spectrometry (Shimadzu GC-MS, GC-17A / QP2010 Plus) with ion source to 220 °C and impact energy of 70 eV, and the same experimental conditions were maintained as in gas chromatography. Fragments were analyzed in the scan range of *m/z* 40-500. The relative amount (%) of each component oil was expressed the peak area as a percentage the total peak area of the oil.

2.5. Component identification

The identification of essential oil components was performed by determination of their retention index (RI), calculated using the equation of Van den Dool & Kratz[11] for each constituent by injecting a series of linear hydrocarbon standards (C_8-C_{40}) and the sample under the same conditions and comparing the result with the tabulated value, followed by confirmation of visual mass spectral patterns reported in the literature[12] and the library's database.[13]

2.6. Larvicidal activity test

Bioassays were developed using the methodology recommended by the World Health Organization.[14-16] The population of *A. aegypti* strain Rockefeller was maintained in the laboratory at 27 ± 1 °C with a relative humidity of 70 ± 5%. Eggs were placed to hatch in a plastic pot with 1 L of dechlorinated water. After hatching, larvae were fed daily with fish food (Nutriflakes, Nutriconpet). The larvicidal activity assay was performed in quadruplicate in 50 mL plastic cups into which were added ten 3rd instar larvae in 10 mL of dechlorinated water. The essential oil was diluted in an aqueous solution of 2% dimethylsulfoxide (DMSO; Isofar) and distributed among the cups at concentrations of 0.08, 0.15, 0.25, 0.30, 0.35 and 0.50 $\mu g.\mu L^{-1}$. Larval mortality was checked after 24 h of exposure to these concentrations the oil. A time course test was performed for the concentration of 0.50 $\mu g.\mu L^{-1}$ (higher mortality in 24 h) where larval mortality was observed 1, 2, 4, 6, 12 and 24 h after exposure to oil and a second test for the concentration of 0.30 $\mu g.\mu L^{-1}$ (LC_{50}) observed 1, 6, 24 and 48 h after exposure to the oil. Parallel controls were included with water and 2% DMSO. The GraphPad Prism® 5.0 program was used to calculate the percent mortality and the LC_{50} (defined as the oil concentration required to kill 50% of the larvae in 24 h) was calculated by Probit analysis using the BioStat professional 5.8.0 program.[17]

3. Results and Discussion

The yield of essential oil from *E. candolleana* leaves was 0.82 ± 0.02%. The chemical composition of the essential oil is shown in Table 1. In all, 28 chemical constituents were identified, corresponding to approximately 94.77 ± 0.36% of the oil's total content. This essential oil is composed primarily of sesquiterpenes (94.21%). The β-elemene component (35.87 ± 0.13%) (a) was identified as the major constituent, followed by δ-elemene (8.28 ± 0.02%) (b) β-caryophyllene (8.15 ± 0.08%) (c) and viridiflorene (6.96 ± 0.05%) (d) (Figure 1). The study by Nakamura *et al*[8] demonstrated a chemical profile similar to that featured in this study, showing sesquiterpenes as the major components identified in the essential oil of *E. candolleana* leaves, but β-elemene was not identified in the chemical composition of the oil with δ-elemene (13.87 ± 0.18%) as the major component. It is considered that variations in the chemical composition of the essential oil of the same species from different regions may be attributed to differences in geographical and climate parameters, such as temperature, altitude, wind direction, rainfall, soil type, etc.[18] These soil and climatic conditions may explain the observed differences between our study and that of Nakamura *et al*.[8] In the previous study, the species of interest was collected in the summer (January), while the one described herein was collected in winter (July); both were from the Atlantic Forest region.

In other species of *Eugenia* genus, accounts of β-elemene as a major component are rare, Ramos *et al*.[19] and Apel *et al*.[20] describe β-elemene (22.1 and 10.6%, respectively) as major constituent in the

essential oil of *E. punicifolia* collected in the Carapebus forest on the southeast coast of Brazil, and essential oil of *E. ramboi* collected in southern Brazil, respectively. Brun *et al*.[21] identified this constituent among the majority, but not as the main essential oil of *E. uniflora* leaves, while Stefanello *et al*.[22] reports the presence of β-elemene as only 1% of the chemical composition of the essential oil from *E. pyriformis*.

Table 1. Average percentage of essential oil components from the leaves of *Eugenia candolleana*

No.[a]	Compound	RI[b]	RI[c]	Relative amount %[d] ± SD[e]	Relative amount %d ± SD[e] Nakamura *et al*.[8]
1	3(*Z*)-Hexenol	854	850	0.05 ± 0.01	
2	Isopentyl acetate	857	869	0.07 ± 0.00	
3	3-*p*-Menthene	990	984	0.08 ± 0.01	
4	1-*p*-Menthene	1010	1021	0.03 ± 0.00	
5	Limonene	1010	1024	0.14 ± 0.02	
6	Allyl Hexanoate	1075	1079	0.09 ± 0.01	
7	Linalool	1103	1095	0.09 ± 0.02	
8	δ-Elemene	1343	1335	8.28 ± 0.02	
9	β-Elemene	1406	1389	35.87 ± 0.13	
10	β-Cariophyllene	1432	1417	8.15 ± 0.08	
11	α-Humulene	1464	1452	1.42 ± 0.02	
12	9-*epi*-(*E*)-Cariophyllene	1484	1464	1.55 ± 0.01	
13	Ishwarane	1493	1465	5.00 ± 0.02	
14	β-Selinene	1502	1489	5.31 ± 0.01	
15	Viridiflorene	1510	1496	6.96 ± 0.05	
16	Germacrene A	1525	1508	4.44 ± 0.03	
17	δ-Amorphene	1529	1511	1.04 ± 0.05	
18	γ-Cadinene		1513		0.90 ± 0.04
19	δ-Cadinene		1519		2.83 ± 0.08
20	Zonarene		1530		0.33 ± 0.00
21	α-Cadinene		1533		0.47 ± 0.00
22	α-Calacorene		1534		0.67 ± 0.00
23	Elemol		1544		0.17 ± 0.00
24	Germacrene B	1569	1559	0.84 ± 0.03	
25	Maaliol		1562		0.78 ± 0.00
26	Spathulenol		1577		4.88 ± 0.02
27	Cariophyllene oxide	1590	1582	1.05 ± 0.03	
28	Globulol	1594	1590	0.74 ± 0.02	5.52 ± 0.02
29	Viridiflorol	1597	1592	0.50 ± 0.01	4.25 ± 0.03
30	Rosifoliol	1607	1600	0.99 ± 0.01	0.95 ± 0.01
31	Guaiol		1600		0.39 ± 0.00
32	β-Atlanol		1607		0.78 ± 0.02
33	1,10-Di-*epi*-cubenol		1611		0.78 ± 0.00
34	Junenol		1614		0.90 ± 0.00
35	1-*epi*-Cubenol		1621		7.59 ± 0.02

36	Muurola-4,10(14)-dien-1β-ol		1624		8.68 ± 0.02
37	Citronellyl pentanoate	1630	1624	0.90 ± 0.04	
38	γ-Eudesmol	1640	1630	0.73 ± 0.03	0.36 ± 0.01
39	*epi*-Cubenol		1631		0.36 ± 0.01
40	*cis*-Cadin-4-en-7-ol	1648	1635	3.99 ± 0.01	
41	*epi*-α-Muurolol		1638		3.50 ± 0.01
42	α-Muurolol		1642		3.53 ± 0.01
43	Cubenol	1662	1645	1.54 ± 0.02	
44	α-Cadinol		1650		5.26 ± 0.01
45	Pogostol	1667	1651	0.43 ± 0.01	
46	(*E*)-10,11-Dihydroathantone		1666		0.53 ± 0.02
47	β-Athantone		1666		0.53 ± 0.02
48	Selin-11-en-4α-ol	1678	1658	4.46 ± 0.01	
49	Eudesma-4(15),7-dien-1β-ol	1682			1.03 ± 0.12
	Chemical group				
	Unsaturated aliphatic alcohol			0.05 ± 0.01	
	Aliphatic esters			0.16 ± 0.01	
	Monoterpene hydrocarbons			0.26 ± 0.04	
	Oxygenated monoterpenes			0.09 ± 0.02	
	Sesquiterpenes hydrocarbons			78.87 ± 0.26	
	Oxygenated sequiterpenes			15.34 ± 0.10	
	Total identified			94.77 ± 0.36	
	Unidentified			5.26 ± 0.28	

[a]Order of elution is given on VF-5MS; [b]RI indicates the retention indices that were calculated against C8-C40 n-alkanes on VF-5MS column; [c]RI indicates values reported in the literature for the VF-5MS; [d]relative amount determined as the peak area relative to the total peak area; [e]Standard deviation.

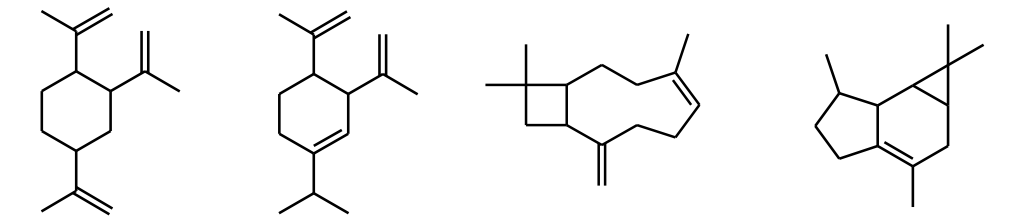

Figure 1. Structures of majoritary substances in essential oil from the leaves of *Eugenia candolleana*

The biological test showed that the percent larval mortality caused by the essential oil of *E. candolleana* reached 100% after 24 h of treatment at concentration of 0.50 μg.μL^{-1}. The concentration of 0.50 μg.μL^{-1} was significantly more efficient than the other concentrations and the control group (Figure 2a). This result is the first report of larvicidal activity of *E. candolleana* against larvae of *A. aegypti*. Based on this result, we evaluated the larvicidal activity of the essential oil of *E. candolleana* through a cronological test, in which larval mortality was analysed 1, 2, 4, 6, 12 and 24 h after

exposure to the oil at a concentration of 0.50 $\mu g.\mu L^{-1}$ (Figure 2b).

We can highlight that the highest concentration that killed 100% of the larvae in 24 h is also able to kill approximately 50% of the larvae between 2 and 4 h (Figure 2b). Larvae from the control groups with water and 2% DMSO displayed zero mortality during the experiment. The DMSO used in the dilution of the oil caused no mortality, indicating that it influenced neither larval development nor death of the larvae in the treated groups. Several studies have been developed with the objective of evaluating the potential insecticide plant species, in the search for new substances that can be used as a vector controls, especially in *A. aegypti*. The most studied family is the Myrtaceae,[23-34] representing 13.5% of active oils, while studies on species of Lamiaceae and Rutaceae represent 10.5% and 8.2%,[35] respectively. According to Sukumar *et al.*,[36] Regnault-Roger[37] and Park *et al.*,[32] Myrtaceae species, have great potential for use as insecticides. Studies by Magalhães *et al.*,[38] Aciole *et al.*[39] and Lima *et al.*[40] obtained results that showed high larvicidal activity of an essential oil rich in oxygenated sesquiterpenes. Lima *et al.*[40] correlated the activity of *L. seriguela* essential oil with high concentrations of these compounds in the mixture. Simas *et al*. [41] observed that sesquiterpenes were more effective than oxygenated monoterpenes and phenylpropanoids against the larvae of *A. aegypti*. As reported by several authors, the oxygenated sesquiterpenes exhibit effective activity against *A. aegypti* larvae, but no reports showed sesquiterpenes hydrocarbons with larvicidal activity, such as those identified in the present study. Usually, the activity of an essential oil correlates with those of its major components. However, these special metabolites, through synergism/antagonism, may facilitate interactions that increase or decrease the larvicidal activity of the oils tested in comparison with the activities of the isolated constituents.[42] According to the data obtained in this study after 24 h, the median lethal concentration (LC_{50} - 24 h) of the essential oil from *E. candolleana* was 0.30 $\mu g.\mu L^{-1}$ (300 $mg.L^{-1}$) (Figure 2c, 2d).

As the WHO has not established standard criteria to determine the larvicidal activity of natural products, we are faced with a conflict in the literature since there is no definite pattern with regard to LC_{50}. Cheng *et al.*[43] consider the following concentration LC_{50} > 100 $mg.L^{-1}$ are not active, LC_{50} < 100 $mg.L^{-1}$ are active, and those with LC_{50} < 50 $mg.L^{-1}$ are highly active. Kiran *et al.*[46] consider compounds with LC_{50} < 100 $mg.L^{-1}$, to be significantly larvicidal. Other authors have developed their own criteria to characterize the power of larvicidal activity of natural products,[23,44,38]. However, Komalamisra et al.[45] considered products with LC_{50} <50 $mg.L^{-1}$ active, those with 50 $mg.L^{-1}$ <LC_{50} <100 $mg.L^{-1}$ moderately active, those with 100 $mg.L^{-1}$ <LC_{50} <750 $mg.L^{-1}$ effective and those with LC_{50}> 750 $mg.L^{-1}$ inactive. Though, when we compare our result with the criterion of Komalamisra et al.[45] our oil is classified as effective.

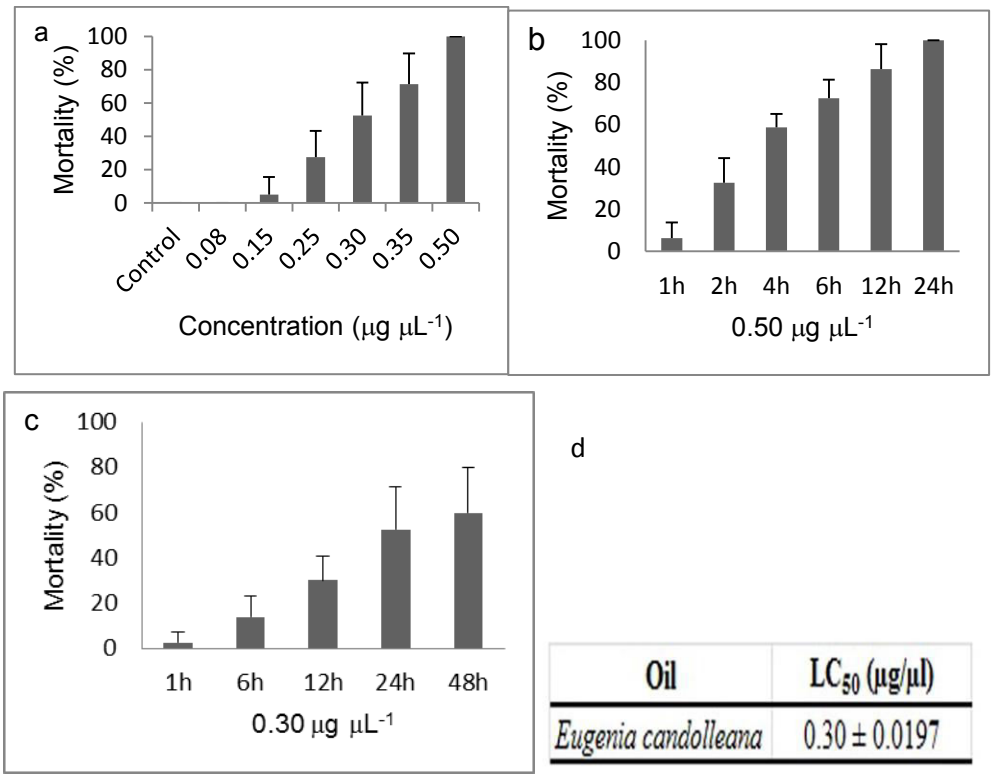

Figure 2. Larvicidal activity of *Eugenia candolleana* oil against the larvae of *Aedes aegypti*

4. Conclusions

The essential oil of *Eugenia candolleana* DC extracted by hydrodistillation showed a rich chemical profile in sesquiterpene hydrocarbons, introducing β-elemene as a major component. This is the first study of larvicidal activity against *A. aegypti*. Larvicidal activity can be attributed to the major component (β-elemene), however more studies with this component are necessary to assign a synergistic or antagonistic effect of essential oil components. This essential oil showed larvicidal activity against *A. aegypti*. The potential lethality of the essential oil, may represent a new possibility for obtaining an active natural product to combat the mosquito that transmits yellow fever and dengue.

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